

IMMUNOGENIC PROPERTIES OF THE PROTEIN COMPONENT OF *Treponema pallidum**

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In a previous paper (Metzger and Podwińska, 1967) evidence was presented that the development of agglutinability which occurs in ageing suspensions of *T. pallidum* (Hardy and Nell, 1955, 1957; Metzger and Podwińska, 1965) proceeds at different rates to different agglutinating antibodies present in syphilitic or immune sera. Most remarkable was the behaviour of the treponemes in a serum that contained antibody to the heat-labile treponeme component only; the agglutinability of the treponemes, suspended in phosphate buffered saline of pH 7.4 and maintained at 4°C., increased sharply in the first days after their extraction from syphilitic testes, reached maximal values between the sixth and the tenth day of storage, and thereafter decreased rapidly so that, as a rule, 14-day-old organisms were practically not agglutinated by this antibody. Moreover, it was found (Metzger and Podwińska, 1968) that treponemes stored longer than 14 days were also incapable of stimulating the production of antibody to the heat-labile antigen in rabbits.

These findings were interpreted as proof that the heat-labile component of *T. pallidum* becomes destroyed during prolonged maintenance, most probably through the action of autolytic enzymes, and is absent from the bodies of stored treponemes.

Further experiments (Metzger and Podwińska, 1968) have shown that this antigen does not resist heating at 56°C. for 1 hr, and is easily destroyed by proteolytic enzymes: trypsin, papain, and pronase, and a number of chemicals, such as formalin, phenol, merthiolate, sodium desoxycholate; only lysozyme and penicillin were found not to affect this component. The destroying effect exerted by proteolytic enzymes upon the heat-labile treponeme antigen has indicated its protein nature.

The present study was designed to investigate

the role of this treponeme component in inducing immunity against syphilitic infection. The results show that the protein antigen of *T. pallidum* is a carrier of immunogenicity.

Materials and Methods

The virulent Nichols strain of *Treponema pallidum* was used throughout.

Adult Danish albino rabbits with non-reactive VDRL and TPI tests were selected for the study; the animals were housed in individual cages out of doors.

The following suspensions of treponemes were administered to the rabbits as immunizing agents:

- (i) Treponemes suspended in phosphate buffered saline of pH 7.4; stored at 4°C. from 7 to 10 days before administration;
- (ii) Treponemes suspended in phosphate buffered saline of pH 7.4 with the addition of penicillin 4 µg./ml.; stored before administration at 37°C. for 20 hrs and at 4°C. for an additional 6 to 9 days;
- (iii) Treponemes suspended in phosphate buffered saline of pH 7.4; stored at 4°C. from 20 to 23 days before administration;
- (iv) Treponemes as under (i), but heated to 100°C. for 1 hr before injection.

The non-infectiousness of the preparations was ascertained by the following experiments:

(1) In initial repeated experiments it was established that treponemes suspended in buffered saline of pH 7.4 and maintained at 4°C. for 96 hrs were constantly unable to produce either symptomatic (lesions) or asymptomatic (no lesions but positive lymph node transfer) infections in rabbits upon intracutaneous injection of 24 million organisms during an observation period of 3 months; treponemes that had been maintained with penicillin added (4 µg./ml.) lost their virulence for rabbits after contact with this antibiotic at 37°C. for as little as 6 hours.

(2) Each batch of treponeme suspensions under (i), (ii), and (iii), prepared anew for every immunization week, was tested for virulence by injecting 0.1 ml. of the suspension containing 29 million organisms at each of four sites on the shaven back of a rabbit, and 0.5 ml.,

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containing 145 million organisms, into each testis of the same rabbit. None of the rabbits developed lesions during a 4-month period of observation, and the lymph gland transfers from these rabbits to other rabbits were negative. All the animals remained sero-negative to VDRL and TPI tests.

(3) Between the 4th and the 5th week after completion of the immunization procedure, the left popliteal node of each animal was removed and emulsified in 1 ml. of 10 per cent. normal rabbit serum-saline; one drop of the emulsion was examined by darkfield microscopy and the rest was injected into one testis of a normal rabbit; the second testis was utilized for lymph node transfer from another rabbit. Microscopical examination of the testes performed one month later did not show the presence of treponemes in any of the testicles.

Immunization Procedure

Only female rabbits were used for the immunization study. The animals were injected intravenously with various lots of treponemal vaccines over a period of 7 weeks. Each rabbit received 1 ml. inoculum containing 180 million organisms into the marginal ear vein four times a week for 3 weeks, and 2 ml. inoculum containing 360 million organisms four times a week for an additional 4 weeks. Thus, the total dose of treponemes for each animal was approximately 8,000 million organisms.

Infectivity Test

5 weeks after completion of the immunization procedure intradermal challenge of 300,000 virulent *T. pallida*, suspended in 0.1 ml. of 10 per cent. normal rabbit serum-saline, was made at each of four sites on the shaven back of each animal. The rabbits were examined daily, and the time when syphilitic lesions appeared at the challenge sites was noted. After 4 months, the rabbits were killed by injection of air into the marginal ear vein. Three to four lymph nodes were then excised from rabbits that failed to develop lesions upon challenge and these nodes were emulsified in 1 ml. of 10 per cent. normal rabbit serum-saline. One drop of the emulsion was examined by darkfield microscopy and the rest was injected into one testis of a normal rabbit; the second testis was utilized for lymph node transfer from another rabbit. The infection was allowed to develop for one month. After that time the rabbits were killed, and the

removed testes were examined microscopically for the presence of treponemes.

The course of immunization and the manner of demonstrating the post-immunization status are shown in Table I.

The presence or absence of the heat-labile protein component in stored treponemes used for the immunization of rabbits was assessed in terms of their agglutinability in an anti-heat-labile antigen serum. The manner of preparing a serum containing antibody to the heat-labile treponeme component only and the technique of the agglutination reaction have been described previously (Metzger and Podwińska, 1968).

Serological Testing

This was performed at the end of the 5-week rest period after completion of the immunization procedure. The VDRL slide flocculation test was carried out as described in the "Manual of Serologic Tests for Syphilis" (1959). The TPI test was performed by the procedure of Nelson and Diesendruck (1951) with the following modifications: the basal medium contained double strength sodium thioglycollate, and bovine serum albumin was replaced with gelatin to a final concentration of 100 mg. per cent.

Results

Clinical Response to Immunization

As can be seen in Table II, none of the sixteen rabbits immunized with non-viable treponemes, being fully agglutinable in an anti-heat-labile antigen serum (Vaccine i), developed lesions at any of the challenge sites. The degree of resistance, however, was not the same in all animals of this group; six rabbits were found to be completely immune, while the remaining ten were considered to be only partially immune because their lymph nodes harboured virulent treponemes that caused infection upon transfer into the testes of normal rabbits.

A high proportion of rabbits immunized with penicillin-killed treponemes having also their heat-labile protein antigen preserved (Vaccine ii)

TABLE I
IMMUNIZATION PROCEDURE AND DEMONSTRATION OF IMMUNITY

Immunization				Interval after Immunization (wks)	Infectivity Test				
Total Dose per Rabbit	No. of Injections	Route	Duration (wks)		Total Dose per Rabbit	Number of Sites Inoculated	Dose per Site	Route	Period of Observation (wks)
8,000 million non-viable <i>T. pallida</i>	28 (4 times a week)	Intra-venous	7	5*	1,200,000 virulent <i>T. pallida</i>	4	300,000 organisms in 0.1 ml.	Intracutaneous	16†

*Followed by lymph node control and serological testing.

†Followed by lymph node transfer from rabbits without clinical manifestations.

TABLE II
ROLE OF PROTEIN ANTIGEN OF *T. PALLIDUM* IN INDUCING IMMUNITY

Vaccine No.	Type of Vaccine	Agglutinability in an Anti-heat-labile Antigen Serum	Results of Infectivity Test			
			Number of Rabbits with Lesions	Number of Positive Challenge Sites	Mean Incubation Period (days)	Number of Rabbits without Lesions but with Positive Lymph Node Transfer
i	Treponemes stored at 4°C. for from 7 to 10 days	1,280-2,560(1)	0/16(2)	0/64(3)		10/16(4)
ii	Treponemes with penicillin added (4 µg./ml.) stored at 37°C. for 24 hrs and at 4°C. for an additional 6 to 9 days	640-2,560	4/11	9/44	25	2/7
iii	Treponemes stored at 4°C. for from 20 to 23 days	< 40-40	6/6	24/24	23	
iv	Treponemes stored at 4°C. for from 7 to 10 days and heated at 100°C. for 1 hr before injection	< 40	12/12	48/48	13	
No vaccine			11/11	44/44	14	

Notes: (1) Reciprocals of highest serum dilution that gave definite agglutination.

(2) Numerator = Rabbits with lesions; Denominator = Rabbits inoculated.

(3) Numerator = Positive challenge sites; Denominator = Sites inoculated.

(4) Numerator = Rabbits without lesions but with positive lymph node transfer; Denominator = Rabbits examined.

acquired a high degree of resistance to the challenge inoculation as indicated by the lack of lesions. Lymph nodes from only two of seven animals that failed to develop lesions were found to be infective. It is interesting to note that a small number of lesions (nine per 44 challenge sites) that occurred in four rabbits of this group appeared after distinctly prolonged incubation times and were atypical: they were small, flat, erythematous lesions without subsequent necrosis and of short duration.

In contrast to the immunizing effect exerted by treponemes having their heat-labile protein component preserved, treponemes that had lost this antigen after prolonged storage (Vaccine iii) or heat treatment (Vaccine iv) failed to protect rabbits against infection. The only sign of resistance was found in rabbits immunized with Vaccine iii, in which the incubation period of the lesions was significantly longer than in the control animals (23 and 14 days respectively).

Serological Response

The post-immunization sera of all the rabbits were strongly positive to the VDRL test with titres ranging from 1:16 to 1:256. However, reagin levels could not be related to the particular treponeme suspension used for immunization or to the immune status of individual animals.

The TPI antibody titres of the sera are given in Table III (overleaf). Rabbits immunized with treponemes lacking the heat-labile protein antigen (Vaccines iii and iv) responded with very low levels of TPI antibody. Sera from rabbits that had been given suspensions of treponemes with the protein antigen preserved (Vaccines i and ii) had average TPI titres much higher than those of the former groups of animals. However, no true correlation could be established between the response of individual animals and their immune status; rabbits whose sera exhibited the same or nearly the same degree of TPI reactivity behaved differently on challenge, being either completely immune or showing asymptomatic or even symptomatic infection.

Discussion

Numerous unsuccessful attempts to induce artificial immunity against syphilitic infection in laboratory animals and humans by the use of killed *T. pallida* have been reported (Turner and Hollander, 1957; Cannefax, 1965; Knox, Dacres, Short, and Glicksman, 1967). The main reason for these failures lies undoubtedly in lack of information on the nature of the immunogenic factor of the treponemes and its situation in the treponemal body.

Tani, Inoue, and Asano (1951) believed that the immunizing component of the treponeme was

TABLE III
POST-IMMUNIZATION TPI ANTIBODY RESPONSE

(1) Vaccine No.	Rabbits showing Reaction to Challenge					
	Immune (2)		Asymptomatic (3)		Symptomatic (4)	
	No. of Rabbits	TPI Titres (5)	No. of Rabbits	TPI Titres	No. of Rabbits	TPI Titres
i	6	230, 260, 340, 580, 1,250, 8,600	10	36%, 84%, 62, 90, 100, 108, 270, 300, 360, 1,200	0	
ii	5	200, 360, 420, 600, 980	2	86, 250	4	50, 170, 460, 1,280
iii	0		0		6	60%, 68%, 88%, 88%, 92%, 96%
iv	0		0		12	8%, 8%, 8%, 12%, 12%, 20%, 28%, 40%, 44%, 46%, 64%, 92%

Notes: (1) See Table II.

(2) No lesions at sites of challenge and lymph node transfers negative.

(3) No lesions at sites of challenge but lymph node transfer positive.

(4) Lesions at one or more challenge sites.

(5) Reciprocals of serum dilutions that immobilized 50 per cent. of the organisms; where percentage marks are given the figures denote the percentage of immobilized treponemes determined by the qualitative TPI test only.

situated deep in the body of the organism and must be "unmasked" by removing a covering material before the component is immunogenic. They postulated that antiformin, used by them for killing treponemes in the vaccine, might be capable of destroying the surface substance.

Miller (1967), on the contrary, was of the opinion that the peripheral layer of the treponemes played an essential role in the induction of immunity. On the basis of immunization experiments, in which treponemes attenuated by γ -irradiation were used as a vaccine, he attributed the immunogenic properties of *T. pallidum* to the irradiation-resistant heat-stable antigens, presumably polysaccharide in nature.

The results reported earlier by McLeod (1962), who failed to immunize rabbits with a desoxycholate-extracted protein-like antigen of *T. pallidum* (TPCF antigen) seem to conform with Miller's theory.

However, the results here presented have clearly shown that the immunogenic activity of treponemes is related to their heat-labile protein component. It was found that non-viable treponemes that had their heat-labile protein antigen well preserved, as evidenced by their full agglutinability in the anti-heat-labile antigen serum, gave rabbits a high degree of protection against challenge inoculation. None of the sixteen animals which received Vaccine i developed lesions at any of the challenge sites. A high degree of immunity, although somewhat lower than in the former group of animals, was also found in rabbits given treponemes stored with the addition of penicillin (Vaccine ii) for the same lengths of

time as the previous ones. It might be argued that the vaccine preparations contained viable organisms which caused infection on repeated injection and, in consequence, the observed resistance of rabbits to challenge was a manifestation of infection immunity. This, however, seems unlikely. The non-infectiousness of the treponeme suspensions used for immunization was ascertained by several appropriate control experiments; none of the batches of the vaccine preparations was found to contain virulent organisms.

In contrast to the immunizing effect produced by treponemes with intact protein antigen, treponemes that had lost this antigen either after prolonged storage (Vaccine iii) or heat treatment (Vaccine iv) failed almost completely to protect rabbits against challenge inoculation.

As already mentioned, evidence was presented in a previous paper (Metzger and Podwińska, 1968) that the heat-labile protein antigen of *T. pallidum* is easily destroyed by even moderate heating (56°C., 1 hr) and by a number of substances that are widely used in laboratories for killing treponemes such as formalin, phenol, and merthiolate; only penicillin was found not to affect this component. However, the present finding that four of the eleven rabbits injected with penicillin-treated treponemes (Vaccine ii) developed lesions, although they were small in number, atypical, and appeared only after a prolonged incubation as compared to those in controls, does appear to indicate that penicillin, or its derivatives, does little damage to the protein antigen. This effect, however, was not reflected in the lessened agglutinability of the treponemes in a

serum that contained antibody to the protein antigen only. Other treponemicidal substances that will not impair the treponeme protein component are now being sought in this laboratory; they will enable the preparation of a suspension of non-viable treponemes with preservation of their immunogenic properties.

These results together with those previously reported (Metzger and Podwińska, 1968) may explain the failure of those investigators who have endeavoured to induce immunity by using treponemes killed by heat or by a variety of chemicals. Organisms so treated were most probably devoid of the protein component which, as has been shown, is a carrier of immunogenicity.

Serological testing performed after completion of the immunization procedure has fully corroborated the findings of other investigators who failed to establish any relationship between the levels of TPI antibody and resistance to infection (Magnuson, Thompson, and McLeod, 1951; McLeod and Magnuson, 1953; Miller, Fazzan, and Whang, 1963). It was particularly interesting to note that rabbits immunized with treponemes that had had their protein antigen preserved responded with much higher TPI titres than those injected with treponemes depleted of this antigenic component. This would indicate that the heat-labile protein antigen of *T. pallidum* plays a predominant role in stimulating the production of immobilizing antibody as was postulated by Hardy and Nell (1957), though objected to by McLeod and Garson (1962) and D'Alessandro (1964).

Summary

Four groups of rabbits were immunized with non-viable *T. pallidum*: two groups received treponemes that had their heat-labile protein component well preserved, as assessed by their high agglutinability in an anti-heat-labile antigen serum; two other groups were given treponemes that had lost this antigenic component after prolonged storage or heat treatment. The total dose, 8,000 million organisms for each rabbit, was given in 28 intravenous injections over a period of 7 weeks. The non-infectiousness of the treponeme suspensions used for immunization was ascertained by several appropriate experiments. 5 weeks after completion of the immunization procedure, intradermal challenge inoculation of 300,000 virulent *T. pallida* was made at each of four sites on the shaven back of each animal.

A high degree of immunity to challenge inoculation was found in rabbits injected with treponemes having their protein antigen preserved. On the other

hand, treponemes deprived of this antigen failed almost completely to confer resistance against infection.

No relationship could be established between the post-immunization immobilizing antibody titres and the degree of resistance.

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Propriétés immunogéniques du composant protidique de *Treponema pallidum*

RÉSUMÉ

Quatre groupes de lapins furent immunisés avec des *T. pallida* rendus avirulents: deux groupes reçurent des tréponèmes dont la protéine thermolabile avait été bien préservée comme le montrait leur fort pouvoir d'agglutination dans un sérum préparé avec un anti-antigène thermolabile; deux autres groupes reçurent des tréponèmes qui avaient perdu ce composant antigénique par long stockage ou par chauffage. La dose totale, 8.000 millions d'organismes pour chaque lapin, fut administrée en 28 injections intraveineuses sur une période de 7 semaines. La non virulence des suspensions

de tréponèmes utilisés pour l'immunisation fut établie par plusieurs expériences appropriées, et, 5 semaines après la fin du dispositif d'immunisation, une inoculation d'épreuve fut effectuée: injection intradermique de 300.000 *T. pallida* virulents à chacun des 4 points choisis sur le dos, rasé, de chaque animal.

Un haut degré d'immunité à cette inoculation fut observé chez les lapins qui avaient reçu des tréponemes

dont le composant protidique avait été préservé. A l'opposé, les tréponèmes pour lesquels ce composant antigénique avait été détruit furent presque complètement incapables de conférer une résistance à l'infection.

Aucune relation ne put être établie entre les titres de l'anticorps immobilisant après l'immunisation et le degré de résistance.